IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

TALECRIS BIOTHERAPEUTICS, INC. and BAYER HEALTHCARE LLC,

Plaintiffs,

v.

BAXTER INTERNATIONAL INC. and BAXTER HEALTHCARE CORPORATION,

Defendants.

BAXTER HEALTHCARE CORPORATION,

Counterclaimant,

v.

TALECRIS BIOTHERAPEUTICS, INC. and BAYER HEALTHCARE LLC,

Counterdefendants.

C. A. No. 05-349-GMS

JURY TRIAL DEMANDED

PUBLIC VERSION

DECLARATION OF BRIAN T. CLARKE IN SUPPORT OF DEFENDANT BAXTER INTERNATIONAL INC. AND DEFENDANT/COUNTERCLAIMANT BAXTER HEALTHCARE CORPORATION'S REPLY IN SUPPORT OF ITS MOTION FOR LEAVE TO FILE AMENDED ANSWER AND COUNTERCLAIM

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Dated: November 27, 2006
Public Version: December 4, 2006

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

TALECRIS BIOTHERAPEUTICS, INC. and BAYER HEALTHCARE LLC, Plaintiffs, v. BAXTER INTERNATIONAL INC. and BAXTER HEALTHCARE CORPORATION, Defendants.	Civil Action No.: 05-349-GMS Jury Trial Demanded CONFIDENTIAL-FILED UNDER SEAL PURSUANT TO PROTECTIVE ORDER
BAXTER HEALTHCARE CORPORATION, Counterclaimant, v. TALECRIS BIOTHERAPEUTICS, INC. and BAYER HEALTHCARE LLC, Counterdefendants.	**PUBLIC VERSION**

DECLARATION OF BRIAN T. CLARKE IN SUPPORT OF DEFENDANT BAXTER INTERNATIONAL INC. AND DEFENDANT/COUNTERCLAIMANT BAXTER HEALTHCARE CORPORATION'S REPLY IN SUPPORT OF ITS MOTION FOR LEAVE TO FILE AMENDED ANSWER AND COUNTERCLAIM

I, Brian T. Clarke, declare:

1. I am an associate at the law firm of Townsend and Townsend and Crew LLP and one of the counsel of record for Defendant Baxter International Inc. and Defendant/Counterclaimant Baxter Healthcare Corporation (collectively "Baxter"). I make this declaration of my personal knowledge.

2. I am a licensed attorney in the State of California, bar number 194234, and certified to practice before the United States Patent and Trademark Office, registration number 45,552.

3. While I am not a registered as a patent attorney with the European Patent Office, I have managed and overseen patent prosecution under the regulations and laws governing the European Patent Office.

4. I recently confirmed with a registered European patent attorney my understanding of the law and basic procedures involved in the making of and defending against arguments for lack of inventive step in European prosecution and opposition practice.

5. I am personally knowledgeable and have confirmed such knowledge that the lack of inventive step in European patent law is analogous to obviousness in United States patent law.

6. I am personally knowledgeable and have confirmed such knowledge that in European prosecution or opposition proceedings an examiner or an attorney will make the case for or against inventive step by identifying a prior art disclosure as "the closest item of prior art," which prior art will serve as a point of reference for comparison to additional prior art references to determine whether the combination provided an obvious solution to the problem solved by the invention.

7. I am personally knowledgeable and have confirmed such knowledge that it is accepted practice in a European opposition to switch from one combination of references to a different combination of references when arguing lack of inventive step.

8. I am personally knowledgeable and have confirmed such knowledge that the accepted practice in Europe often necessitates switching the designation of the closest prior art from one of the references in a first combination of references to one of the references in a second combination of references.

9. I am personally knowledgeable and have confirmed such knowledge that changing the designation of the closest prior art from one reference to another reference in an inventive step analysis does not necessarily inform the reader about the relative materiality between the two references designated as the closest prior art; it is merely a

procedural device to facilitate discussion of a particular combination of references for making an inventive step determination in Europe.

10. A true and correct copy Ng *et al.*, "Process-Scale Purification of Immunoglobulin M Concentrate" *Vox Sang* 65:81-86 (1993) is attached hereto as Exhibit 1.

11. A true and correct copy of U.S. Patent No. 5,256,771 to Tsay is attached hereto as Exhibit 2.

12. A true and correct copy of the Consent Order entered October 31, 2006 in *Baxter Healthcare SA and Baxter Healthcare Pharmaceutical Limited and Baxter Healthcare Limited v. Bayer Corporation and Bayer Healthcare LLC and Talecris Biotherapeutics Inc.* HC 06C 01197 is attached hereto as Ex. 3.

13. A true and correct copy of a letter from Jeffrey Bove, counsel for Plaintiffs, to Susan Spaeth, counsel for Defendants, dated July 25, 2006 is attached hereto as Ex. 4.

14. A true and correct copy of a letter from Priya Sreenivasan, counsel for Defendants, to Jeffrey Bove dated August14, 2006 is attached hereto as Ex. 5.

15. A true and correct copy of pages 222, 290-295, and 298-305 of the deposition transcript from the October 2, 2006 deposition of William Alonso Volume II is attached hereto as Ex. 6.

16. A true and correct copy of pages 104-107 of the September 29, 2006 deposition of Susan Trukawinski Volume I is attached hereto as Ex. 7.

17. I am aware that Priya Sreenivassan issued a subpoena for documents from and a deposition of Paul K. Ng on September 25, 2006.

18. I am informed that neither counsel for Bayer or Talecris identified themselves as representing Paul Ng prior to the deposition.

19. I have reviewed portions of the deposition transcript for Paul Ng.

20. A true and correct copy of pages 1-5 and 63-65 from the October 3, 2006 deposition of Paul Ng is attached hereto as Exhibit 8.

21. From the deposition transcript of Mr. Ng I have determined that the deposition took place on October 3, 2006. Ex. 8, p. 4:13-14.

22. From the deposition transcript of Mr. Ng I have determined that Gabrielle Ciuffreda of the law firm Ropes & Gray and Chris Jeffers of the law firm Connely, Bove, Lodge & Hutz, both of whom represent one or both of the Plaintiffs in this matter, represented Mr. Ng. Ex. 8, p. 5:5-7.

23. From the deposition transcript of Mr. Ng I have determined that Mr. Jeffers asked questions of Mr. Ng, and that these questions were directed towards the content of Ng *et al.*, "Process-Scale Purification of Immunoglobulin M Concentrate" *Vox Sang* 65:81-86 (1993). Ex. 8, p. 63-65.

24. A true and correct copy of pages 9 and 12 of the August 1, 2006 deposition of James Giblin is attached hereto as Ex. 9.

I declare under penalty of perjury under the laws of the United States that the foregoing is true and correct. Executed this 27th day of November, 2006, in Palo Alto, California.

Brian T. Clarke

Public Version: December 4, 2006

EXHIBIT 1

Original Paper

Vox Sang 1993;65:81–86

Process-Scale Purification of Immunoglobulin M Concentrate

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Abstract

An IgM concentrate was purified from Cohn fraction III. Efficiency of euglobin precipitation was shown to be controlled by pH and ionic strength. Prekallikrein activator activity in the product was insignificant. Overall yield from the octanoic acid supernate and purity of the concentrate were 66 ± 8 (n = 16) and 50 ± 5% (n = 16), respectively. Solvent-detergent treatment to inactivate lipid-enveloped viruses was demonstrated and implemented into the process. Process studies to control residual virucidal agents and C4a generating activity are presented.

Introduction

The Cohn plasma fractionation process yields a waste product, fraction III [1]. Fraction III is known to be a source of an immunoglobulin fraction enriched in IgM. Octanoic acid was employed to precipitate lipids and lipoproteins present in fraction III [2]. The proteins remaining in the supernatant were then precipitated with ethanol. The product thus prepared contains prealbumin, 20–25% IgM and 15–20% IgA. Combining this method with Aerosil and ion exchange resulted in a preparation that contains 4 mg IgM/ml or five times the concentration of IgM in normal plasma [3, 4]. Due to its pentamer structure, IgM is particularly suited to agglutinate bacteria. In a mouse model it was demonstrated that the IgM-containing preparation protects mice against *Salmonella* infection more effectively than a conventional intravenous IgG preparation [3]. In a recent clinical study, such a preparation containing 10–15% IgM was shown to be effective in the therapy of gram-negative sepsis [5]. Mortality in IgM-treated patients was 4 versus 32% in the control group. Thus, it is reasonable to believe that preparations of higher purity than the 10–15% IgM concentrate commercially available in Europe would be desirable from the standpoint of more rapid infusion and improved efficacy thereby increasing its therapeutic potential. In this paper, we will present a method of obtaining such a preparation. Specifically, we will discuss the classical euglobulin precipitation method where precipitation is controlled by pH and ionic strength. All procedures in the present study were designed to be compatible with production-scale sanitary application where continuous centrifugation is still a widely accepted technique in protein purification. We will also discuss process control with regard to inactivation on lipid-membrane-coated viruses and product complement activity by its ability to generate C4a. Classical pathway activation usually requires an involvement of immunoglobulins, immune complexes or immune aggregates. C4a generation signals particularly of the immune system in such a capacity as mediating inflammatory reaction [6]. And in addition, comparison of the physical and chemical properties of C4a with those of C3a and C5a establishes a high degree of structural similarity [7]. Therefore, an assay to measure C4a generating ability was developed to determine an acceptable level of anticomplement activity on IgM solution intended for infusion

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into an animal [8]. A potential vasodilator, prekallikrein activator (PKA), is also evaluated in the product. The proposed process has been scaled up for pilot plant operations with reproducible product characteristics.

Materials and Methods

Purification Method

Figure 1 shows a flow diagram of the purification process. Fraction III paste used in this study was obtained frozen from a plasma fractionation plant (Clayton, N.C.). The paste (30 kg) was allowed to thaw overnight at 4°C, after which it was solubilized in 12 vol of 0.05 *M* Na acetate buffer, pH 3.75, for 4 h at 25°C. The solution was then stirred with 1.5% (v/w) octanoic acid, pH 4.66, for 4 h, cooled to 5°C and settled overnight. Precipitate was removed by centrifugation in two tubular bowl centrifuges (Sharples AS16). The supernatant was clarified further by passage through 0.1-µm nominal filter cartridge. The clear solution was concentrated 10-fold and diafiltered against at least 5 vol water for injection. The operation was carried out in a 120-ft^2 Romicon ultrafiltration system with a nominal molecular weight cut-off value of 100,000. Completeness of diafiltration was checked by sampling the retentate and measuring the conductivity using an Orion conductivity-meter. Typically, the undiafiltered sample was 2.6 mmho/cm and the diafiltered sample was 0.05 mmho/cm. A sample was taken at this point for optimization studies. The salt-poor solution, typically at an A_{280} of 40–60, was then treated with a mixture of 0.3% tri-(n-butyl) phosphate (TNBP) and 1% Tween 80, pH 4.6–4.8, for at least 8 h at 25°C for viral inactivation. The TNBP/Tween-80-treated solution was diluted with water for injection to a conductivity of less than 0.1 mmho/cm. Addition of water was needed because of the slight increase of ionic strength following pH adjustment to 4.6–4.8. Residual reactants from the viral inactivation step were removed and euglobulins were recovered by precipitation twice at pH 6.7 using 0.5 *M* NaOH, added at a rate of 20–30 ml/min. The paste recovered in a tubular bowl centrifuge (Sharples AS16), was prepared as a 5% protein solution in 10% maltose, pH 4.25 and held at 5°C. Several batches could then be combined and heated at 50°C for 1 h. Each batch was then diafiltered against at least 5 vol of 0.0025 *M* Na acetate, pH 4.25, in a Romicon ultrafiltration system with a nominal cut-off value of 500,000. The solution was formulated in 10% maltose, sterile filtered and filled. To provide an accelerated sterility check, the final containers were held at 25°C for 21 days prior to storage at 2–10°C.

C4a Generating Activity

The radioimmunoassay kit for human complement C4a des Arg (Amersham Inc., Arlington Heights, Ill., USA) was used. A pooled preparation of serum from at least 3 donors served as the complement source for activation. Modified from the procedure established by Wagner and Hugli [6], C4a generated was determined by measuring the ability of the sample to compete with a fixed amount of ^{125}I-labeled C4a des Arg tracer for a limiting quantity of rabbit anti C4a des Arg. All samples were tested at a constant concentration of 1.46 mg IgM/ml serum. The C4a generating activity was expressed in µg/ml of serum. Since there is a dilution of potential activated compounds in preparing the sample for this assay, a control sample, commercial intravenous γ-globulin solution, was prepared similarly.

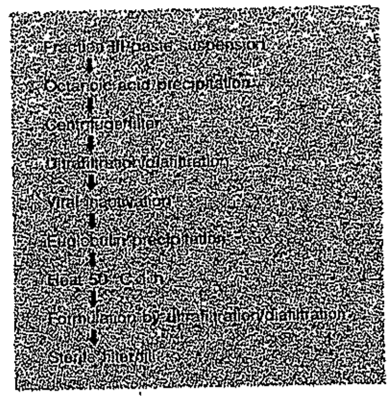

Fig. 1. IgM concentrate purification flow sheet.

Protein Concentration

In process protein concentration was determined by absorbance measurement at 280 nm and an extinction coefficient of 13.8. Total protein was calculated from the sample volume multiplied by protein concentration.

Quantitation of IgA, IgG and IgM

Quantitation of each immunoglobulin was determined by immunoprecipitation, with detection by infrared nephelometry. An automated Behring nephelometer (Behring Diagnostics, Somerville, N.J., USA) measured the sample turbidity due to light-scattering immune complexes between the antigen and a specific antiserum. Detailed description of the method is available in the manufacturer's instruction manual.

Quantitation of Tween 80

Sample was first deproteinized by 95% ethanol. Tween 80 remains in the supernate. Ethanol was evaporated at 50°C and Tween 80 was redissolved in distilled water. The polyethoxylated compounds in Tween 80 formed a blue-colored complex with ammonium cobaltothiocyanate reagent, which was soluble in dichloromethane. Optical density of the blue dichloromethane solution was measured at 620 nm. This optical density value was proportional to the concentration of Tween 80.

Viral Inactivation Studies

Vesicular stomatitis virus (VSV), a lipid-enveloped virus was chosen for this study. Titers were measured and expressed in terms of tissue culture infectious doses as a 50% endpoint per ml ($TCID_{50}$/ml) [9].

Quantitation of PKA

PKA activity was measured by a two-stage assay. It is based on the initial conversion by PKA of a partially purified prekallikrein substrate to kallikrein. This proteolytic enzyme is then assayed by its esterase action on the synthetic substrate, α-N-benzoyl *L*-arginine ethyl ester. The rate of α-N-benzoyl *L*-arginine ethyl ester hydrolysis is monitored by the change in optical absorbance of the mixture at 253 nm. The activity is expressed as a percentage of a Bureau of Biologics reference PKA preparation.

Results and Discussion

Effects of pH in Euglobulin Precipitation

Proteins become positively or negatively charged on either side of the isoelectric point, and these forms are more soluble than the electrically neutral molecule. The isoelectric point of IgM concentrate has been determined by isoelectric focusing to cluster around neutrality [unpubl. data]. Accordingly, IgM would precipitate from a solution at a pH range close to 7. In the experiment shown in figure 2, the pH of a diafiltered octanoic acid supernate (conductivity = 0.05 mmho/cm) at pH 4.6 was adjusted by 0.5 *M* NaOH to between 6.4 and 8.5. The precipitate was recovered and solubilized in an equal volume of water. Based on the A_{280} measurement, total protein was plotted against the pH at which precipitation occurred. The shape of the curve determined from 3 experiments reflects isoelectric behavior of the proteins with insolubility occurring between 6.4 and 7.5. From these results, it followed that the best recovery of the euglobulin is obtained at this pH range.

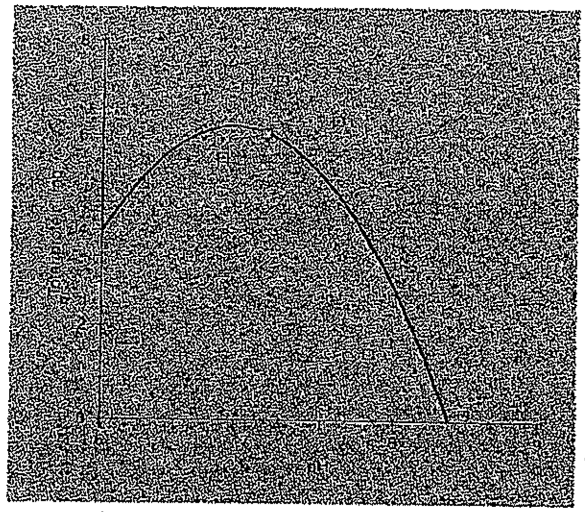

Fig. 2. IgM precipitation as a function of pH.

Effects of Ionic Strength in Euglobulin Precipitation

The diafiltered octanoic acid supernate was precipitated at pH 7.1 in solutions of four different ionic strengths, as designated by the molar concentrations of NaCl in figure 3. Each solution was centrifuged to remove the precipitate. As measured by infrared nephelometry, the amount of IgM, IgG and IgA remaining in the supernate represented the solubility of each globulin. It is evident that reduced solubility is associated with lower NaCl concentration, i.e., lower ionic strength. This relationship conforms to the Debey-Huckel theory [10] where solubility of globulin increases upon the addition of salts. Our results suggest that solubilities of polymeric immunoglobulins, both IgM and IgA, are very similar. Additionally, ionic strength exerted a more pronounced effect on the solubility of IgG. Thus, preferential precipitation of IgG from IgA and IgM occurs at low NaCl concentration.

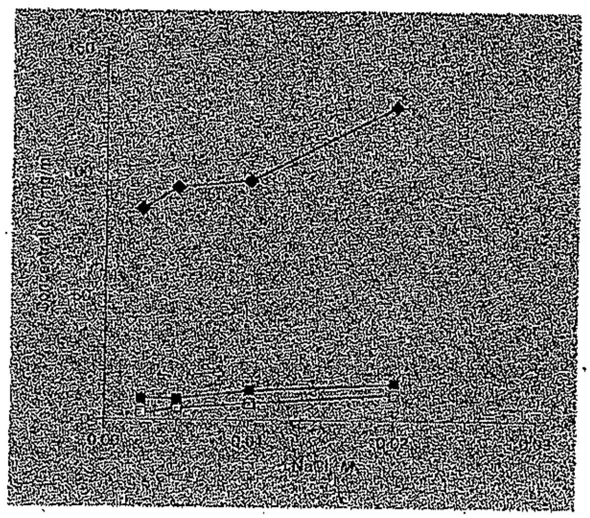

Fig. 3. Effects of ionic strength on the supernatant concentration after euglobulin precipitation. □ = IgM; ◆ = IgG; ■ = IgA.

Marker Virus Inactivation

Conditions for viral inactivation were similar to those developed by the New York Blood Center [9]. The inactivation of VSV added to an IgM solution containing 0.3% TNBP and 1.0% Tween 80 was studied over a pH range of 4.25–5.1. Results from a typical experiment are shown in figure 4. Relative to the untreated control titer of $10^{6.75}$ $TCID_{50}$/ml, minimal inactivation was seen at pH 5.1 for 3 h. This is contrary to previous observations with detergent/solvent treatment on AHF solutions at neutrality [9]. The difference could be due to much higher protein concentrations (A_{280} greater than or equal to 40) according to our purification scheme. It is in agreement with our in-house data that VSV infectivity is stabilized by proteins [unpubl. obs.]. Between pH 4.25 and 4.8, greater than 10^4 $TCID_{50}$/ml reduction in titer was achieved. At pH 4.25 and

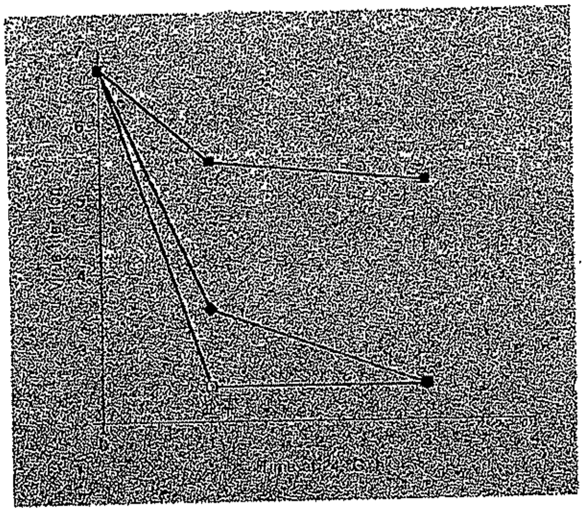

Fig. 4. Inactivation kinetics of VSV in IgM containing 0.3% TNBP and 1.0% Tween 80. □ = pH 4.25/4.4; ◆ = pH 4.8; ■ = pH 5.1.

Table 1. Preparation of 50% IgM concentrate

Product profile					
Lot No	IgA mg/ml	IgG mg/ml	IgM mg/ml	% of IgM in product	overall yield of euglobulin ppt %
1	12.1	18.2	31.1	51	63
2	11.7	16.3	41.7	60	64
3	15.2	22.0	28.8	44	50
4	13.8	22.4	27.1	43	62
5	11.7	20.0	35.0	52	66
6	10.8	17.4	25.2	47	70
7	10.8	25.6	29.1	44	79
8	11.7	19.5	30.2	49	60
9	10.6	24.3	26.5	43	78
10	9.4	17.9	34.3	56	68
11	11.0	17.9	37.7	57	68
12	12.0	18.3	24.6	45	63
13	9.5	22.4	30.5	49	75
14	14.8	18.2	37.1	53	71
15	8.8	22.4	33.1	51	69
16	12.7	17.4	32.7	51	55

pH 4.4, inactivation was complete to the limit of detection after 1 h, the first time point assayed. While the use of pH 4.25 is preferred in terms of faster viral inactivation, it must be counterbalanced by the unacceptable increase in ionic strength when euglobulin precipitation is carried out by NaOH addition. Preliminary consideration thus suggested that a pH of 4.6–4.8 is a reasonable compromise, thereby achieving viral inactivation in 3 h and maintaining low ionic strength in the subsequent steps. It is noteworthy that our inactivation studies demonstrated that the acid condition alone was not the mechanism of inactivation since titers of the untreated controls at pH 4.6–4.8 were unaffected after 4 h.

Product Composition and Overall Yield

IgM concentrations in 16 preparations can be seen in table 1. IgM content in the concentrate was 50 ± 5% (n = 16). Each batch of fraction III paste was derived from a pool of over 3,000 plasma donors and, as a consequence, may exhibit little variation in the resulting immunoglobulin concentrations. Overall yield from the octanoic acid supernate to the final product averaged 66 ± 8% (n = 16).

Residual Reactants from Viral Inactivation

Considerable retention of TNBP/Tween 80 in the product was shown if precipitation was carried out only once after the viral inactivation step. TNBP, having a molecular weight of 266.3, is easily removed when the product is diafiltered. However, Tween 80 exists as detergent miscelles and could bind to protein molecules by hydrophobic interactions [11]. Successful removal of traces of Tween 80 (<25 ppm) is accomplished by reprecipitation after solubilizing the protein in a large volume of water. This volume was determined to be 40 vol equivalents of the protein precipitate (fig. 5). It can be observed from this figure that fewer volumes were not consistently effective in removing the residual Tween 80 from the protein solution.

Reduction of C4a Activity

When purified from human serum, globulin proteins (IgM, IgG, IgA) contain enzymatic as well as complement active proteins and other proteins as contaminants. One of the marker components in the complement-cascade chosen for this study is C4a generating activity. Heat has been shown to reduce this activity in globulins. Reduction of this activity is a function of temperature and length of heating [12]. This is consistent with the observation that

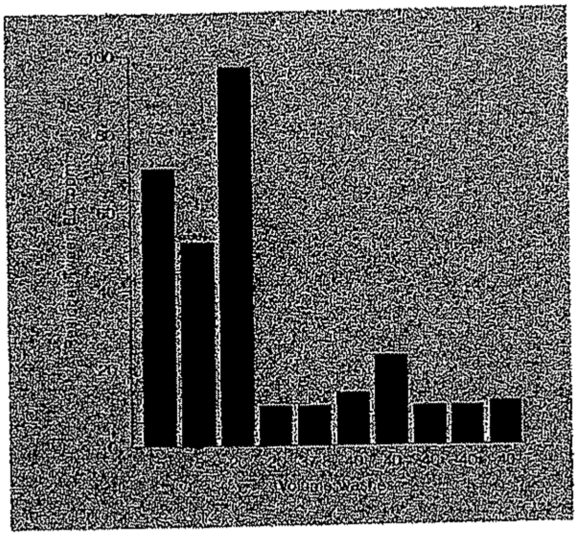

Fig. 5. Residual Tween 80 as a function of washes.

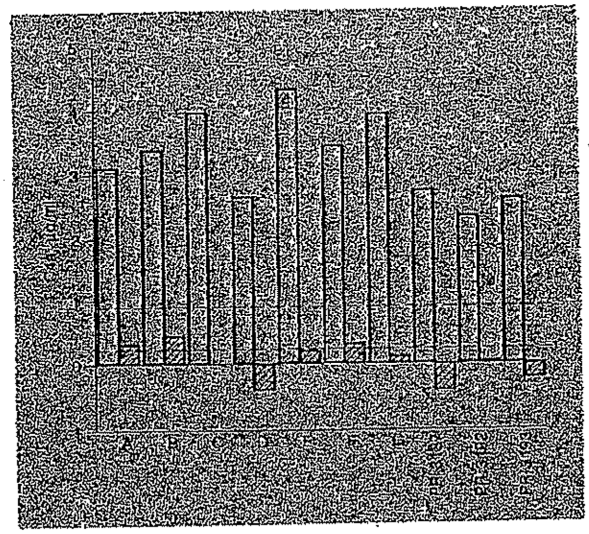

Fig. 6. C4a reduction across heating. ☐ = Preheated C4a activity of each lot in x-axis (μg/ml); ▨ = postheated C4a activity of each lot in x-axis (μg/ml); ---- = C4a activity in IGIV 0.1–0.93 μg/ml.

anticomplementary activity in intravenous human γ-globulin is reduced by incubating the solutions at pH 4 and 37 °C for 8 h [13]. Upon heating at 50 °C for 1 h, high C4a generating activity in unheated IgM preparations was greatly reduced to levels equal to or below those of a commercial intravenous γ-globulin preparation (fig. 6). Results obtained from HPLC and efficacy studies in mice indicated that the immunoglobulin was protected from denaturation due to heating [12].

PKA Activity in IgM Concentration

The most desirable IgM solution would be one that contains no potentially vasoactive agents. Lefer [14] has reviewed many cardioinhibitory factors, most of which are small peptides with molecular weights less than 10,000. If such factors are present, it is likely that the two diafiltration steps would aid in the removal of inhibitory effects. One identifiable vasodilator is pKA [15] which could trigger the generation of bradykinin. With a goal of below 20% of a bureau of Biologics reference PKA preparation, the study reported here demonstrates that fraction III can be processed to IgM concentrate which has a low amount of PKA (table 2).

Table 2. PKA activity in IgM concentrate

Product lot No.	PKA activity % of reference
A	<1
B	8
C	1
D	<1
E	<1
PR 3187	<1
PR 3192	<1

Conclusion

We have reported a processs aimed at the manufacture of an IgM concentrate from Cohn fraction III paste. Efficiency of euglobulin precipitation was largely determined by pH and ionic strength. Solvent-detergent treatment, the method of choice for viral inactivation in many plasma derivatives, was successfully applied to this product with a marker virus, VSV. IgM in the concentrate represents 50% of the immunoglobulins present. Clearly, process

reproducibility was demonstrated by meeting all criteria with regard to residual reactants, IgM concentration, PKA activity and C4a generating activity. While this kind of data is useful in guiding the development of an industrial process, it should be noted that significant issues associated with preclinical studies such as development of a suitable animal model must still be resolved. The capacity to produce large quantities of purified IgM should facilitate further biochemical studies of structure and function of this immunoglobulin.

Acknowledgment

The authors wish to acknowledge Neal Cheung on the C4a assay, and Dr. Rae Victor and staff on the analytical support. Jack Smiley's input on euglobulin precipitation and Dr. G. Mitra's guidance on the project are also appreciated.

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EXHIBIT 2

US005256771A

United States Patent [19]
Tsay et al.

[11] **Patent Number: 5,256,771**
[45] **Date of Patent: Oct. 26, 1993**

[54] **HEAT TREATMENT OF IGM-CONTAINING IMMUNOGLOBULINS TO ELIMINATE NON-SPECIFIC COMPLEMENT ACTIVATION**

[75] Inventors: Grace C. Tsay, Walnut Creek; Gary Jesmok, Pinole, both of Calif.

[73] Assignee: Miles Inc., Berkeley, Calif.

[21] Appl. No.: 504,161

[22] Filed: Apr. 3, 1990

[51] Int. Cl.[5] A61K 39/395; C07K 3/12; C07K 15/06
[52] U.S. Cl. 530/390.5; 424/85.8
[58] Field of Search 424/89.8; 530/387, 389, 530/427, 387.1, 389.1, 390.5; 914/12; 514/2

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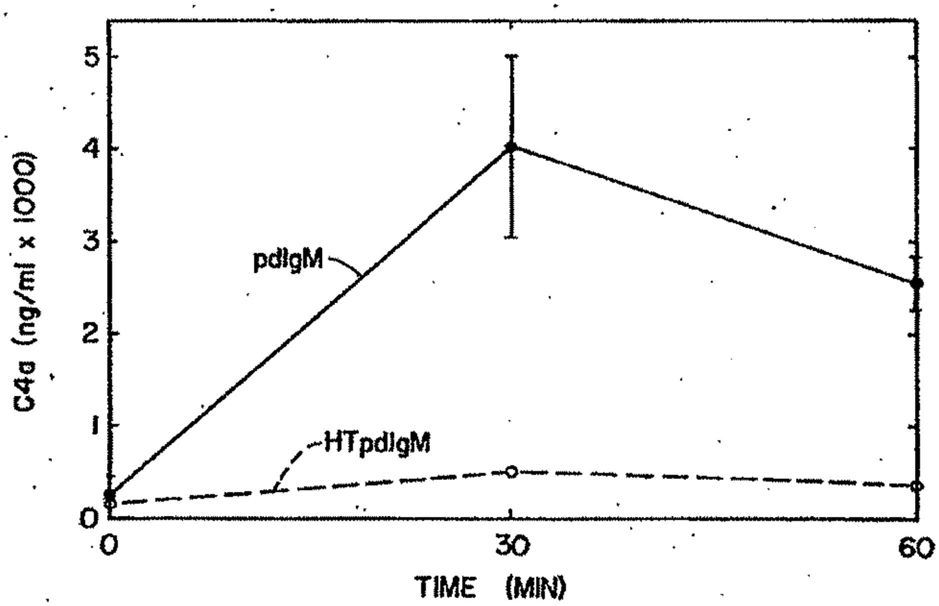
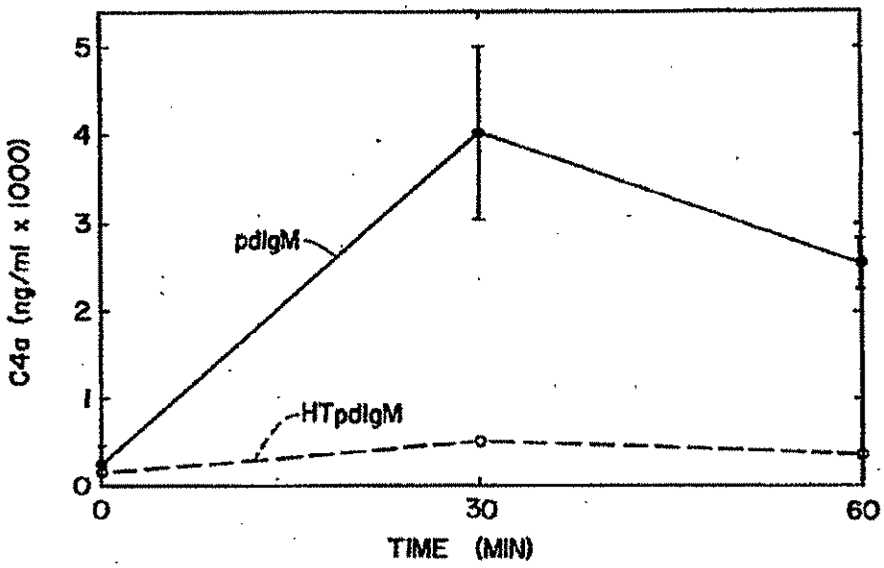
Primary Examiner—Jeffrey E. Russel
Attorney, Agent, or Firm—James A. Giblin

[57] **ABSTRACT**

Mild heat-treatment of IgM antibody concentrates diminishes the potential to induce non-specific complement activation without significant loss of normal immunologic effector functions. These IgM immunoglobulin concentrates retain specific antigen binding properties and activate complement specific antigen binding properties and activate complement when bound to antigen. Preferred product includes at least 20% by weight IgM in an IgM/IgG antibody mixture. Heating is done at a temperature within the range of about 40° C. to 62 ° C., preferably about 45° to 55° C., in a solution having an acid pH (preferably 4.0 to 5.0) for at least about 10 minutes.

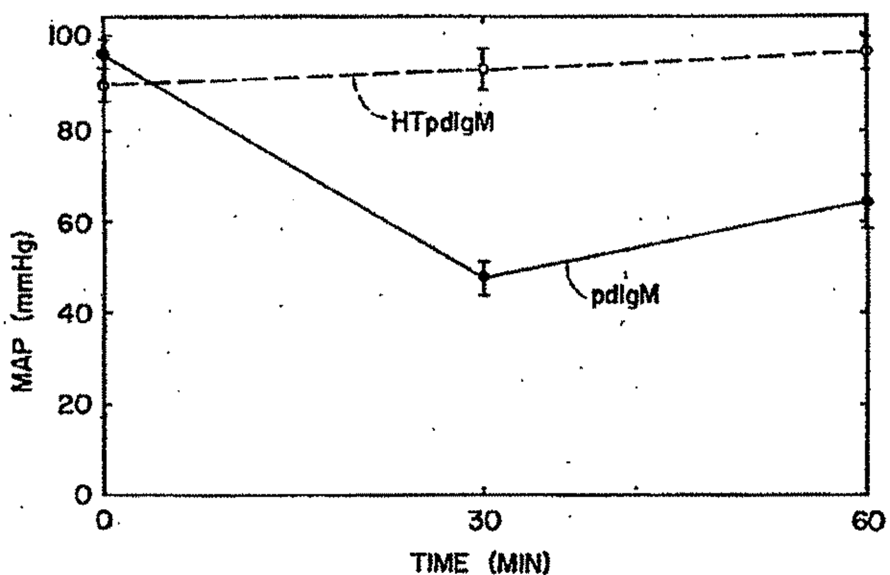
3 Claims, 3 Drawing Sheets





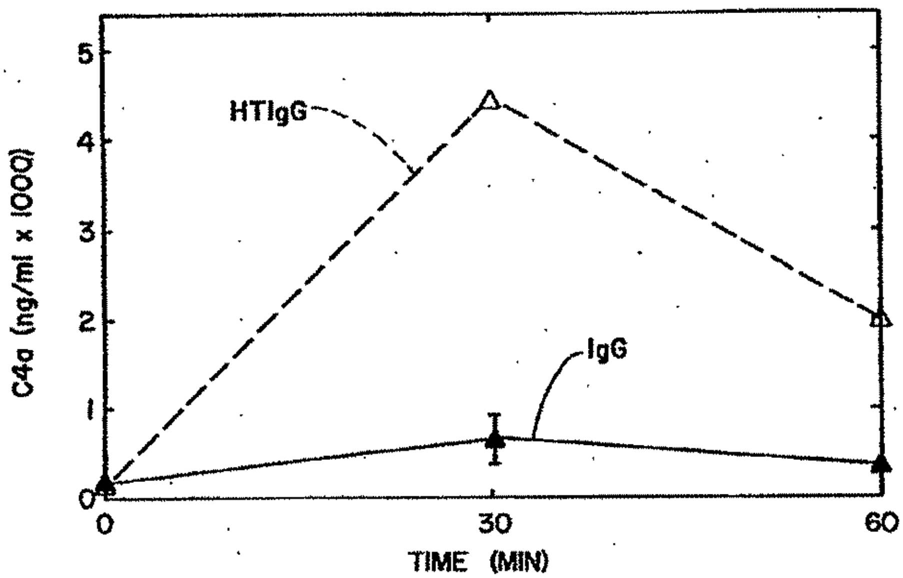
C4a (ng/ml x 1000)
5
4
3
2
1
0
pdIgM
HTpdIgM
0
30
60
TIME (MIN)

FIG._1.



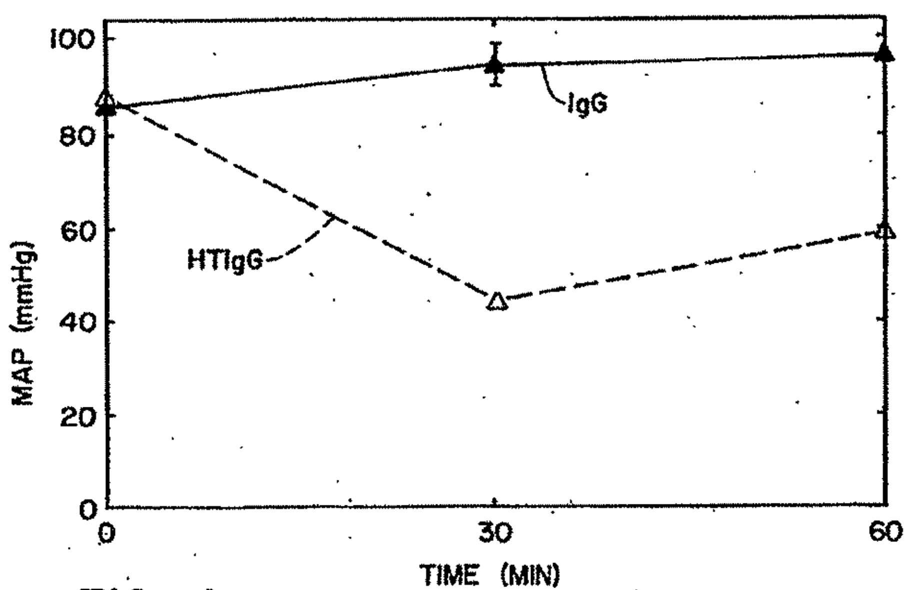
MAP (mmHg)
100
80
60
40
20
0
HTpdIgM
pdIgM
0
30
60
TIME (MIN)

FIG._2.



C4a (ng/ml x 1000)
5
4
3
2
1
0
HTIgG
IgG
0
30
60
TIME (MIN)

FIG._3.



MAP (mmHg)
100
80
60
40
20
0
IgG
HTIgG
0
30
60
TIME (MIN)

FIG._4.

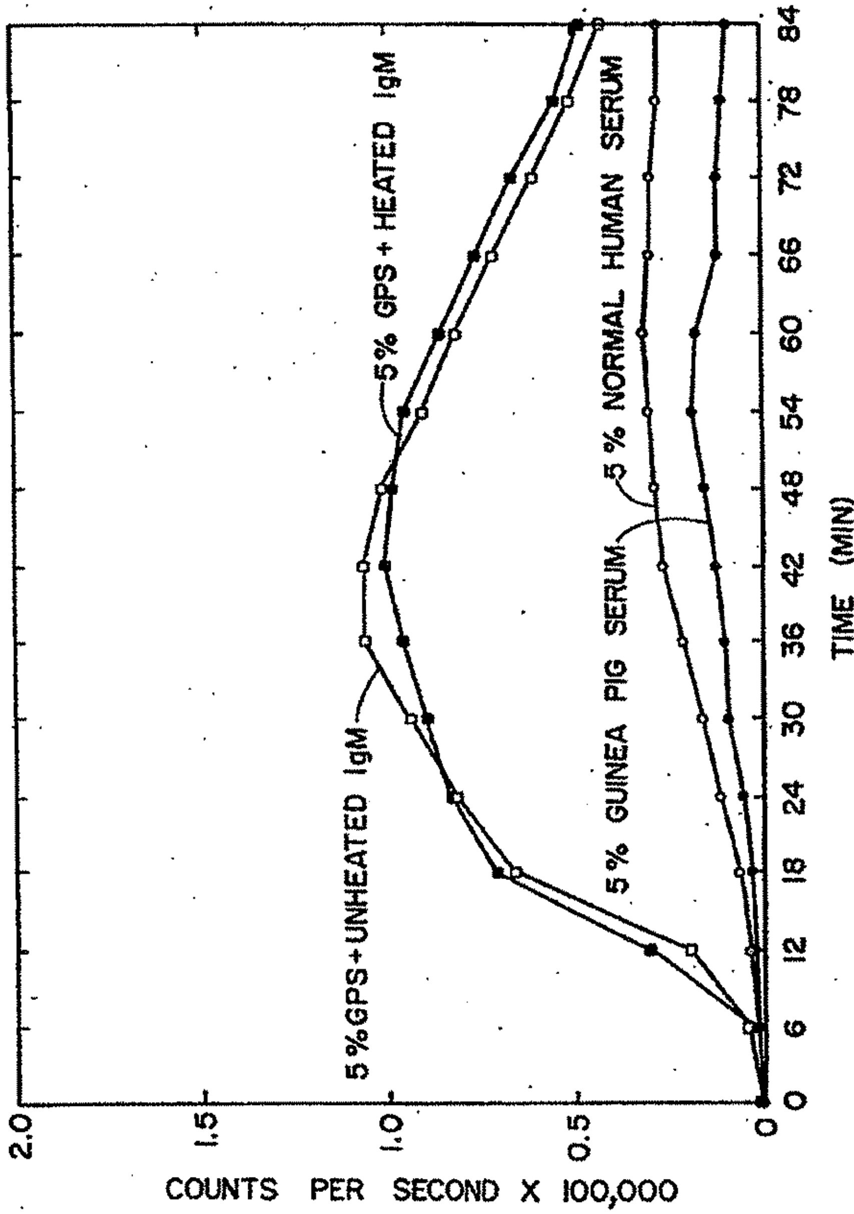

5% GPS + HEATED IgM
5% GPS + UNHEATED IgM
5% NORMAL HUMAN SERUM
5% GUINEA PIG SERUM
COUNTS PER SECOND X 100,000
2.0
1.5
1.0
0.5
0
TIME (MIN)
0
6
12
18
24
30
36
42
48
54
60
66
72
78
84

FIG._5.

HEAT TREATMENT OF IGM-CONTAINING IMMUNOGLOBULINS TO ELIMINATE NON-SPECIFIC COMPLEMENT ACTIVATION

BACKGROUND OF THE INVENTION

1. Field

This disclosure is generally concerned with therapeutic antibody or immunoglobulin preparations and specifically with therapeutic immunoglobulin preparations that include at least some antibodies of the IgM type.

2. Prior Art

Antibodies may be classified according to a well known typing system (i.e. IgM, IgG, IgA, IgD, IgE) and, in case of IgG, according to sub-types (i.e. IgG_1, IgG_2, IgG_3, and IgG_4).

Commercially available immunoglobulin preparations (known as immune serum globulin or ISG) commonly consist mainly of antibodies of the IgG type with the distribution of IgG sub-types approximating that found in human plasma. Typically, the amount of IgM in such preparations, if present at all, is relatively small.

IgM is a well known 19S immunoglobulin which comprises about 7% of the immunoglobulins found in man. IgM antibodies are said to have an antibody valence of at least five and they are the earliest antibodies generated in an immune response. Although IgM antibodies tend to be very effective, especially in combating bacterial infections, they have a relatively short in vivo half life of about five days. Further, IgM antibodies tend to aggregate and are relatively difficult to stabilize, especially in purified form.

To date, the only known commercial intravenous (IV) product having significant amounts of IgM antibody is a product known as Pentaglobin TM, available from Biotest, GmbH, of West Germany. The use of that product appears to be described in articles by K.D. Tympner, et al, "Intravenous IgM-Application," Mschr. Kinderheilk. 123,400-401 (1975) and by K.N. Haque, et al "IgM-Enriched Intravenous Immunoglobulin Therapy in Neonatal Sepsis" Am. J. Dis. Child. 142, 1293-1296 (1988). That product comprises, on a percent by weight total protein basis, about 76% IgG, about 12% IgA and about 12% IgM.

It has been thought that the use of larger amounts of IgM in an ISG product could lead to adverse reactions. For example, it is known that IgM is many times more potent than IgG in activating the complement cascade in an immune reaction. This is because only one molecule of IgM bound to an antigen will activate complement whereas two or more molecules of IgG must be bound to an antigen in close association to each other to activate complement.

It appears that the very production methods used in preparing IgM-enriched products may limit the amount of IgM available due to degradation reactions. See, for example, U.S. Pat. No. 4,318,902 to W. Stephen, describing the use of β-propriolactone to make an IgM enriched product IV administrable. Hence, for whatever reason, even though IgM is recognized as very effective, it has not appeared in any commercially available intravenously useful ISG product at an amount greater than about 12% by weight total protein. Although a 20% by weight IgM product has been available, in the past (Gamma-M-Konzentrat, Behringwerke AG, Marburg, Germany), it has been made for and limited to intramuscular (not IV) applications.

Various purification schemes have been suggested for plasma-derived IgM and, more recently, monoclonal-derived IgM. In the case of plasma-derived IgM, it has been known since the 1940's that alcohol fractionation techniques could be used to obtain a relatively concentrated IgM from what is known as Cohn Fraction III. See also, for example, the above-cited U.S. Pat. No. 4,318,902 (and the cited references) to W. Stephen concerned with the use of beta-propriolactone to make a concentrated (12%) IgM suitable for intravenous (IV) administration. In addition, see EPO application 0 038 667 of Miura et al (IgM acylation). Other IgM purification or preparation techniques are disclosed by U. Sugg et al, Vox Sang. 36:25-28 (1979); M. Steinbach et al, Preparative Biochemistry 3 (4), 363-373 (1973) and A. Wichman et al, Biochem. Biophys. Acta 490:363-69 (1977). For a variety of technical reasons, plasma derived IgM has been relatively difficult to purify and the highest known purity to date (used in analytical purposes) is about 90% IgM, by weight.

In addition to the above problem associated with IgM-rich preparations, it has been observed that the preparations in use tend to generate what is known as non-specific complement activation. Non-specific complement activation refers to the initiation of the complement cascade even in the absence of antibody-antigen complexing. This phenomenon is often associated with the infusion of aggregates of immunoglobulins. Non-specific complement activation is to be avoided since it may cause undesirable side effects such as hypotension. Specific complement activation, on the other hand, is desirable and it occurs only after the immunoglobulin(s) has bound to, for example, the antigenic surface of a microorganism in the bloodstream.

It has been reported by S. Barandun et al "Intravenous Administration of Human Gamma-Globulin," Vox Sang 7, 157-174 (1962) that human gamma-globulin for intravenous administration heated at 37° C. at pH 3.8-4.0 for 24 hours, followed by pH adjustment to 7.0, resulted in a reduction of anticomplementary activity (AC) measured by complement fixation test. However, this treatment for longer periods of incubation resulted in high anticomplementary activity due to the formation of aggregated gamma-globulin. These authors did not demonstrate retention of specific complement activity by the heated immunoglobulin when bound to antigen. Furthermore, no demonstration of in vivo safety was reported by these authors. In addition, M. Wickerhauser et al "Large Scale Preparation of Macroglobulin," Vox Sang 23, 119-125 (1972) demonstrated that IgM concentrates prepared by PEG precipitation had high anticomplementary activity (AC) by standard complement fixation test and this AC ativity was reduced 10 fold by incubating the IgM concentrate at pH 4.0 at 37° C. for 8 hours followed by readjustment to neutral pH. Similar to the previous paper (Vox sang 7, 157-174 (1962), these authors did not assess the specific complement activating potential of the heated IgM concentrate, nor did they assess safety in any animal model.

We have now found that the problem of non-specific complement activation associated with IgM or IgM rich immunoglobulin preparations can be minimized (without losing specific complement activation) in a relatively simple and surprising way.

SUMMARY OF THE INVENTION

Our method of substantially eliminating non-specific complement activation in an IgM-containing immunoglobulin preparation while retaining specific complement activation effector functions comprises the step of subjecting the preparation to a gentle heating step under conditions sufficient to eliminate the non-specific complement activation while not adversely affecting the normal biological activity or antigen binding ability of the IgM antibody. To do this, we have found that the heating step should be at a temperature ranging from about 40° to 62° C., preferably about 45° to 55° C. for at least about 10 min. and the preparation should be in an aqueous solution having an acid pH ranging preferably from about 4.0 to 5.0. To date, the preferred temperature appears to be at or very close to 50° C. for at least about 30 minutes.

Our improved product comprises an immunoglobulin preparation which includes at least some measurable antibodies of the IgM type. On a dry weight basis, a preferred product comprises at least 20% antibodies of the IgM type, the remainder of the antibodies being mainly of the IgG type. Trace amounts (less than 20% by weight) of other types may be present. Details of our preferred product and processes are given below.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 represents plasma $C4_a$ anaphylatoxin levels in monkeys infused with plasma derived IgM (pdIgM) or heat-treated plasma derived IgM (HT pdIgM).

FIG. 2 represents mean arterial blood pressure (MAP) measurements in monkeys infused with plasma derived IgM (pdIgM).

FIG. 3 represents plasma $C4_a$ anaphylatoxin levels in monkeys infused with native intravenous gamma globulin (IgG) or heat-treated intravenous gamma globulin at neutral pH (HTIgG).

FIG. 4 represents mean arterial blood pressure (MAP) measurements in monkeys infused with native intravenous gamma globulin (IgG) or heat-treated intravenous gamma globulin at neutral pH (HTIgG).

FIG. 5 represents ability of unheated or heated IgM to promote phagocyte chemiluminescence against *E. Coli* 0.50:kL bacteria.

SPECIFIC EMBODIMENTS

Work in our laboratory has demonstrated a reproducible adverse response elicited by infusion of IgM-enriched, IgG immunoglobulin concentrates in the pentobarbital-anesthetized cynomolgus monkey. That product consisted of about 50% by weight IgM on a dry weight basis, the remaining being IgG. The mixture was given IV as a 5% aqueous solution. The rate of administration was 1 mg/kg/min (IgM) to a total dose of 50 mg/kg. A major component of the adverse response was a severe decline in arterial blood pressure. In attempting to understand the mechanism of the adverse effect, we demonstrated that heat-aggregated IgG prepared at neutral pH (not acid pH) when infused in the monkey elicited effects remarkably similar to those observed following the infusion of IgM-enriched, IgG immunoglobulin concentrates. Since both the IgM-enriched, IgG immunoglobulin concentrates and aggregates of IgG formed at neutral pH are capable of activating the classical pathway of complement, we hypothesized that complement activation is associated with elicitation of the adverse effect in the cynomolgus monkey. The classical complement pathway is described in Inflammation: Basic Principles and Clinical Correlates Complement: Chemistry and Pathways, pp 21–53, the teachings of which are incorporated herein by reference (Raven Press, NY, N.Y., 1988).

The complement system functions primarily as an effector mechanism in the immune defense against microbial infection. The activated products of the complement system, attract phagocytic cells and greatly facilitate the uptake and destruction of foreign particles by opsonization. There are two pathways for activating complement, the classical pathway and the alternate pathway. Activation of the classical pathway is initiated by antigen-antibody complexes or by antibody bound to cellular or particulate antigens. The alternate pathway is activated independent of antibody by substance such as bacterial wall constituents, bacterial lipopolysaccharides (LPS), cell wall constituents of yeast (zymosan) and Fungi. It is thought that the alternate pathway provides protection against infection prior to an immune response whereas the classical pathway is important after antibody production has occurred.

Activation of the blood complement system generates bioactive peptide fragments called anaphylatoxins. Complement *4a* (C4a) anaphylatoxin is a split product of C4 (MW 8740). When C1q is activated by antigen-antibody complexes or aggregates, the C1 complex splits C4 into C4a and C4b allowing C4b to bind to the activating surface while C4a anaphylatoxin is released into plasma. Recent developments in analytical biochemistry have provided techniques which permit the measurement of plasma C4a by radioimmunoassay. See, for example, U.S. Pat. No. 4,731,336 and European Patent 97,440 both to P.S. Satoh.

Determination of $C4_a$ levels in plasma provides direct information regarding activation of the classical complement cascade in vivo. Furthermore, the induction of C4a generation in vitro, by various immunoglobulin preparations using human serum as the complement source, is correlated with in vivo complement activation in the monkey following infusion of the immunoglobulins.

In the studies described herein, we determined whether adverse effects (hypotension) elicited by IgM-enriched, IgG immunoglobulin concentrates and/or heat-aggregated IgG formed at neutral pH are associated with elevated levels of plasma C4a. In addition, non-specific activation of complement (classical pathway) induced by the immunoglobulin preparations was assessed by C4a generation in vitro.

Using these assay systems, we, furthermore, demonstrate that mild heat-treatment of IgM-enriched, IgG immunoglobulin concentrates diminishes C4a generation in vitro and correspondingly this mild heat-treatment diminishes adverse side effects (hypotension) associated with parenteral (IV) administration in the non-human primate. Finally, we demonstrate that the mild heat-treatment process step did not significantly effect the antigenic determinants of either IgM or IgG or the specific antigen binding sites; thus the effector functions of the immunoglobulin are unaltered. Retention of the desired specific complement activation properties of the immunoglobulin was confirmed in subsequent opsonic studies.

Methods

Adverse effects (hypotension) induced by the various immunoglobulin preparations were assessed in the cy-

nomolgus monkey. The monkeys were anesthetized by an intramuscular injection of Ketamine hydrochloride (5 mg/kg). Following intubation, anesthesia was maintained by intravenous pentobarbital sodium (5–10 mg/kg as needed). Catheters were inserted in the femoral artery and vein for measurement of mean arterial blood pressure and parenteral administration of the immunoglobulin preparations, respectively.

For the IgM enriched, IgG immunoglobulin concentrates we used an infusion rate of 1 mg/kg/min (IgM) up to a total dose of 50 mg/kg. This rate and dose resulted in severe hypotension within 30 minutes (data to be presented).

Blood pressure measurements were taken from the femoral artery over a 90 minute time period since we have demonstrated that adverse effects, if they result, will occur within this time frame. $C4_a$ anaphylatoxin measurements were performed on plasma from whole blood (anticoagulated with Citrate) obtained at 0, 30, 60 and 90 minutes. The samples were stored at −70° C. C4a measurements were made by radioimmunoassay with kits from Amersham International (Arlington Heights, Ill.).

Definitions

As used herein, the expression antibody (or immunoglobulin) preparation means a collection of therapeutic antibodies comprising at least about 20% by weight of antibodies of the IgM type, the remaining antibodies, if present, being mainly antibodies of the IgG type with trace amounts other types such as IgA, etc. The individual antibodies can be obtained from a variety of sources such as plasma (as described above, for example) or from cell culture systems (e.g. monoclonal antibodies from hybridomas or transformed cell lines). In the examples below, our enriched IgM antibody preparation comprised on average about 30% to 50% by weight antibodies of the IgM type, the remaining antibodies being mainly of the IgG type.

Non-specific complement activation means the activation of the complement cascade by immunoglobulin in the absence of antigen.

Minimal non-specific complement activation means, the generation of less than about 1.0 ug/ml $C4_a$ in an in vitro assay in the absence of antigen. Alternatively, minimal non-specific complement activation means an amount of $C4_a$ generation within about 100% of the amount of $C4_a$ generated using a liquid IGIV at pH 4.25 as a control.

Specific complement activation means the activation of the complement cascade by immunoglobulin (of the IgM or IgG type) in the presence of antigen.

Substantially no loss of specific complement activation, as applied to an IgM enriched antibody preparation, means the antibody preparation is capable of binding to antigen and activating the classical pathway of complement in vitro or in vivo.

Materials

Pd IgM Immunoglobulin Concentrate Preparation

Pd IgM immunoglobulin concentrates were isolated from Cohn fraction III paste (45 kg) suspended in 12.5 volumes of 0.05M acetate buffer pH 3.5–4.0 and mixed at room temperature for 2–3 hours. To the mixture was added 2.0% of caprylic acid by vol/wt at pH 4.8 to remove lipoproteins and prekallikrein activator (PKA) by centrifugation. The extracted caprylate supernatant, after diafiltration and ultrafiltration through PM-30, resulted in low conductivity of 0.03–0.06 mm ho/cm at pH 4.8 Virus inactivation was achieved with 0.3% TNBP/1% Tween-80 at 24° C. for more than 6 hours The caprylate supernatant was precipitated with buffer system such as tris (0.0101 vol. of 1M Tris pH 7.8) or imidazole buffer (0.005 vol. of 1M imidazole pH 7.8) sterile water, adjusting pH to 4.0–4.8 with acetic acid, and further diafiltered/ultrafiltered against water, then adding solid glycine to a final concentration of 0.25M glycine, pH 4.0–4.8. The Pd IgM immunoglobulin concentrates resulted in low PKA (less than 10% of reference) and less than 5% aggregate determined by high pressure liquid chromatography (HPLC). The final IgM-enriched product consisted of 50–60% IgM, 30–40% IgG, 3–5% IgA, on a dry weight basis, in a total 5% protein aqueous solution.

Heat-Aggregated IgG Preparation

A 5% solution of IGIV (Lot #2855-IIB) was used as an appropriate antibody control. A heat-aggregated IgG solution was prepared from the 5% IGIV solution by heating at 62° C. for 1 hour (pH 7.0). Another heat-aggregated IgG solution was prepared from the 5% IGIV solution by heating at 62° C. for 2 hour (pH 4.25).

Heat Treated IgM, IgG Preparation

The heat-treatment of the IgM, IgG preparation in water or glycine (pH 4.0–4.8) ranged from 37° C. to 62° C. for periods of 10 minutes to 8 hours to determine the optimal mode for the treatment.

Assay Methods

Aggregate Determination by High Pressure Liquid Chromatography (HPLC)

Aggregate formation in the native IgM and IgG preparations or induced by heating was determined by high pressure liquid chromatography with TSKG 4000 SWXL gel (7.8×300 mm, 8 μm particle size, Toyo Soda Corporation, Japan) and eluted with 0.05M sodium acetate, 0.20M sodium sulfate, pH 5.0.

Biological and Functional Activity Determination of IgM Immunoglobulin Concentrates

1. Antigenic Determinants of IgM-Enriched, IgG by Radial Immunodiffusion (RID)

The concentration and antigenic determinants of IgM and IgG were determined by radial immunodiffusion (RID) with quiplate system from Helene Laboratories (Beaumont, TX). This method provides an indirect assessment of the integrity of the antibody.

Specific Antigen Binding Sites Study by ELISA against $Ps.IT_4LPS$

The biological activity of IgM immunoglobulin concentrates and mild heat treated IgM concentrates was determined by enzyme-linked immunosorbent assay (ELISA) to quantitate IgM binding to $Ps.IT_4LPS$ (lipopolysaccharide) and to assess the integrity of specific antigen binding sites. 10 μg of *P. aeruginosa* immunotype 4 LPS in 0.06M sodium carbonate buffer pH 9.5 were coated to Immulon 1 plates (Dynatech Lab) at 37° C. for 3 hours. Each well of the plates was washed twice with PBS—0.05% Tween buffer. The standard Pseudomonas monoclonal antibody and unheat/heat treated IgM concentrates were diluted in 0.01M Tris buffer pH 7.8 containing BSA and added to the plates incubated at room temperature overnight. Each well was washed three times with PBS - Tween buffer. Goat

anti-human IgM alkaline phosphatase conjugate (Hy-Clone, Logan, Utah) was added to the wells, incubated at room temperature for 4 hours and the wells were washed five times with PBS - Tween buffer. P-Nitrophenyl phosphate in diethanolamine pH 9.8 substrate solution was added to each well at room temperature for 30 minutes and the A405nm/450nm was read.

2. Specific Complement Activity Determination by Phagocytic Assay

A phagocytic assay was employed to determine the opsonic activity (specific complement activity) of mild heat treated IgM immunoglobulin concentrates. The phagocytic assay employed bacteria (*E. coli* 050:kl) and human phagocytes (PMNs) suspended in tissue culture fluid. The bacteria to PMN ratio was 20 to 1 and 5% (vol/vol) guinea pig serum (GPS) served as complement source. 2.5 ul of IgM concentrates was added to the total assay mixture (500 ul) and incubated at 37° C. for 100 minutes. An aliquot of the assay mixture was added to 9 vol. of distilled water to lyse PMNs and surviving bacteria were enumerated by duplicate agar plate counts.

4. In vitro and in vivo Non-specific Complement Activation assessed by Anaphylatoxin ($C4_a$) Generation

The ability of various immunoglobulin preparations to activate the classical pathway of complement in vitro was assessed by incubation of the respective preparations (1.47 mg IgM or IgG/ml serum) with human serum at 37° C. for 20 minutes and determining the resultant generation of $C4_a$ levels by radioimmunoassay (RIA). The RIA kits were obtained from Amersham (Arlington Heights, Ill.).

Systemic complement activation in vivo was assessed by measuring plasma $C4_a$ levels following parenteral administration of the various immunoglobulin preparations in the monkey. Antibodies raised against human $C4_a$ (RIA kits) partially cross react with monkey $C4_a$, approximately 60%.

Results

Table 1 describes the immunoglobulin preparations used in the initial experiments designed to examine the hypothesis that non-specific complement activation may be associated with adverse cardiovascular events following intravenous infusion. To test this hypothesis various immunoglobulin preparations were assayed for their ability to activate the classical pathway of complement in vitro by measuring $C4_a$ generation employing human serum as the complement source. Mean arterial blood pressure in the cynomolgus monkey was measured over a 90 minute period following infusion of the immunoglobulins in order to assess cardiovascular safety. Plasma $C4_a$ levels were also measured in order to ascertain complement activation following infusion.

In Vitro Data

A 5% IGIV immunoglobulin (control) solution at 0.2M glycine pH 4.25 did not cause appreciable $C4_a$ generation in vitro when incubated with human serum (<1%, 0.23 ug/ml). The enriched pd IgM, prepared by euglobulin precipitation in a similar glycine buffer pH 4.25, caused substantial $C4_a$ generation in vitro (4.5 ug/ml). To prepare aggregated IgG, a well known activator of complement, we heated the 5% IGIV protein solution at pH 7.0 at 62° C. for 1 hour. This heat treatment resulted in a solution which had 19% pentamer aggregates yet retained over 72% of its antigenic determinants as assessed by RID. This immunoglobulin solution also generated a substantial amount of $C4_a$ (14.0 μg/ml) when incubated with human serum. However, the same IGIV solution when heated at pH 4.25, although generating 58% aggregates (size less than pentamer), did not generate significant amounts of $C4_a$ in vitro (0.56 ug/ml). This IGG solution also lost over 80% of its antigenic determinants as measured by RID.

TABLE 1

Characteristics of Immunoglobulin Preparations

Antibody	Lot #	Buffer	Heat (hrs) 62° C.	RID IgM mg/ml	RID IgG mg/ml	% Aggregates <Pentamer	% Aggregates >Pentamer	$C4_a$ Generated in vitro (Human) Serum) (ug/ml)
IGIV (5%)	2855-11-B	0.2M Glycine pH 4.25	0	0	57.0	0	0	0.23
pdIgM	3747-82-E	0.2M Glycine pH 4.25	0	36.0	26.2	0	6.0	4.5
IGIV (HT)	18053-79-8	0.2M Glycine pH 7.0	1	0	40.9	11.0	19.0	14.0
IGIV (HT)	18053-66-2	0.2M Glycine pH 4.25	2	0	10.2	58.0	0	0.56

These results demonstrate that both pdIgM, IgG immunoglobulin concentrates and heat-aggregated IGIV at pH 7.0 induced substantial $C4_a$ generation in vitro while native IGIV and heated IGIV at pH 4.25 generated non-significant quantities of $C4_a$ anaphylatoxin.

It was important to determine whether these in vitro measurements of non specific complement activation were associated with adverse cardiovascular effects in the cynomolgus monkey when the immunoglobulin solutions were intravenously infused.

TABLE 2

Acute Effect of Immunoglobulin Preparations on MAP and Plasma $C4_a$ Anaphylatoxin Levels in the Monkey

Antibody	Rate	Dose		Time (min.) 0	30	60	90
IGIV (5%)	10 mg/Kg/min	500 mg/Kg	MAP (mm Hg)	85 ± 5	97 ± 4	96 ± 7	97 ± 6
N = 3			$C4_a$ (ng/ml)	192 ± 91	601 ± 95	385 ± 51	392 ± 180
pdIgM	1 mg/Kg/min	50 mg/Kg	MAP (mm Hg)	97 ± 4	47 ± 3	67 ± 6	64 ± 10
N = 5			$C4_a$ (ng/ml)	253 ± 43	4048 ± 1000	2562 ± 370	611 ± 305
IGIV (5%)	1 mg/Kg/min	20 mg/Kg	MAP (mm Hg)	99 ± 5	47 ± 6	57 ± 7	63 ± 7
pH 7.0 Heated			$C4_a$ (ng/ml)	135 ± 38	4160 ± 268	3100 ± 536	1558 ± 131
N = 3							

TABLE 2-continued

Acute Effect of Immunoglobulin Preparations on MAP and Plasma $C4_a$ Anaphylatoxin Levels in the Monkey

Antibody	Rate	Dose		Time (min.) 0	30	60	90
IGIV (5%) pH 4.25 Heated N = 3	10 mg/Kg/min	500 mg/Kg	MAP (mm Hg)	87 ± 3	85 ± 14	97 ± 7	99 ± 6
			$C4_a$ (ng/ml)	155 ± 17	535 ± 51	372 ± 100	207 ± 30

Table 2 and FIGS. 1-4 presents the in vivo results with these respective immunoglobulin preparations. These results demonstrate that the immunoglobulin preparations which generated substantial $C4_a$ levels in vitro i.e., pdIgM and heat-aggregated IGIV at pH 7.0, caused severe hypotension in the cynomolgus monkey and elevated plasma $C4_a$ levels, while those immunoglobulin preparations which did not generate substantial $C4_a$ in vitro i.e., native IGIV and heat aggregated IGIV at pH 4.25, did not cause hypotension in the cynomolgus monkey and did not greatly increase plasma $C4_a$ levels. Thus, the in vitro assessment of $C4_a$ generation by the various immunoglobulin preparations appeared to be associated with adverse cardiovascular effects in vivo following intravenous infusion.

TABLE 3a

Characteristics of Heat Treated PdIgM, IgG Immunoglobulin Concentrate

Antibody	RID IgM	RID IgG	% Aggregates >Pentamer	$C4_a$ Generated in vitro (Human Serum) ug/ml
pd IgM (pH 4.25) (HT 62° C., 2 hrs)	mg/ml 6.58	12.81	47.0	0.27

TABLE 3b

Acute Effect of Heat Treated PdIgM, IgG Immunoglobulin Concentrate on MAP and Plasma $C4_a$ Anaphylatoxin Levels in the Monkey (N = 3)

Antibody	Rate	Dose		Time (min.) 0	30	60	90
PdIgM (pH 4.25) (HT 62° C. 2 hrs)	2 mg/kg/min	100 mg/kg	MAP (mm Hg)	90 ± 3	94 ± 4	98 ± 4	97 ± 5
			$C4_a$ (ng/ml)	155 ± 19	530 ± 25	372 ± 75	251 ± 60

Since an IGIV protein solution heated at pH 4.25 did not cause substantial $C4_a$ generation in vitro and did not cause hypotension when infused intravenously, we reasoned that perhaps heating the pdIgM, IgG immunoglobulin concentrate at pH 4.25 would diminish the non-specific complement activating potential of the IgM, without adversely affecting the IgG which is present in the solution. That is, heating IgG at acidic pH did not result in a solution which activated complement in vitro and did not have adverse effects when infused in the cynomolgus monkey. To test this hypothesis we initially heated the phIgM, IgG immunoglobulin solution at 62° C. for 2 hours and evaluated its $C4_a$ generating potential in vitro. This solution did not generate significant amounts of $C4_a$ in vitro (0.27 ug/ml) and did not cause hypotension or substantial increases in plasma $C4_a$ when infused in the cynomolgus monkey, Table 3a, 3b and FIGS. 1, 2.

These results demonstrate that heating (62° C. for 2 hours) an IgM, IgG immunoglobulin concentrate at acid pH (4.25) produces a protein solution which has dramatically diminished non-specific complement activating potential in vitro and does not cause hypotension when infused in the cynomolgus monkey. However, this particular heat treatment (62° C. for 2 hrs) resulted in a loss of more than 80% of the IgM antigenic determinants and a greater than 47% pentameric aggregate formation, Table 3.

Thus, although, this heat-treatment diminished the adverse cardiovascular effects associated with intravenous administration, it also appeared to diminish the effector functions of the immunoglobulin. We, therefore, sought to define more closely an optimal heating temperature and incubation time which would result in an IgM, IgG immunoglobulin concentrate which had minimal non-specific complement activating potential while retaining relevant biologic effector functions, i.e., antigen binding, opsonization, etc.

During this evaluation, a number of conditions were examined. Table 4 summarizes data concerning the effect of temperature and incubation time on $C4_a$ anaphylatoxin generation in vitro.

TABLE 4

Effects of Temperature and Incubation Time on C_{4a}* Generation in vitro and IgM Antigenic Determinants of IgM, IgG Immunoglobulin Concentrates (3747-82-E, pH 4.42)

Incubation Time (Min)	62° C. C_{4a} µg/ml	62° C. IgM mg/ml	55° C. C_{4a} µg/ml	55° C. IgM mg/ml	52° C. C_{4a} µg/ml	52° C. IgM mg/ml	50° C. C_{4a} µg/ml	50° C. IgM mg/ml	45° C. C_{4a} µg/ml	45° C. IgM mg/ml	40° C. C_{4a} µg/ml	40° C. IgM mg/ml
0	10.41	35.82	10.41	35.82	10.41	35.82	10.41	35.82	10.41	35.82	10.41	35.82
10	0.49	17.42	1.08	33.49	2.43	35.14						
20	0.49	14.58	0.51	31.21	1.85	35.14	5.41	35.82				
30	0.62	10.68	0.35	26.77	1.45	35.14						
40		-	0.60	26.77	1.24	35.14	2.88	35.82				
60	0.07	4.9	0.48	22.50	1.09	35.39	2.12	33.49	5.08	35.68	12.25	35.68
120			0.50	12.54	0.60	35.00	1.49	33.49	4.35	35.68	7.41	35.68
180					0.82	33.41	0.77	32.28	3.25	35.68	5.24	35.68
240							0.67	24.40	2.74	35.68	5.24	35.68
300							0.86	24.40	3.86	35.68	5.60	35.68

TABLE 4-continued

Effects of Temperature and Incubation Time on C_{4a}* Generation in vitro and IgM Antigenic Determinants of IgM, IgG Immunoglobulin Concentrates (3747-82-E, pH 4.42)

	62° C.		55° C.		52° C.		50° C.		45° C.		40° C.	
Incubation Time (Min)	C_{4a} μg/ml	IgM mg/ml	C_{4a} μg/ml	IgM mg/ml	C_{4a} μg/ml	IgM mg/ml	C_{4a} μg/ml	IgM mg/ml	C_{4a} μg/ml	IgM mg/ml	C_{4a} μg/ml	IgM mg/ml
480									4.13	35.68	5.26	35.68

*Control (no exogenous immunoglobulins) $C4_a$ levels have been subtracted from all reported values.

PdIgM, IgG immunoglobulin concentrates (50% IgM, pH 4.42) heated at 62° C. for 10 minutes caused non-significant $C4_a$ generation in vitro (0.49 ug/ml) but approximately 50% of IgM antigenic determinants were lost. Heating the IgM, IgG immunoglobulin concentrate at 55° C. for 30 minutes decreased $C4_a$ generation to 0.35 ug/ms in vitro and the IgM immunoglobulin retained more than 75% of its antigenic determinants. Heating at 52° C. for 120 minutes decreased $C4_a$ generation to 0.60 ug/ms and immunoglobulin retained more than 98% of its antigenic determinants. Heating at 50° C. for 180 minutes decreased $C4_a$ generation to 0.77 ug/ml and the immunoglobulin retained more than 92% of its antigenic determinants. Immunoglobulin heated at 45°–37° C. retained substantial $C4_a$ generating potential (>4 ug/ml) and did not demonstrate any decrease in IgM antigenic determinants.

We next examined the effects of pH, IgM concentration and incubation times on $C4_a$ generation in vitro, Table 5. Temperature was held constant at 50° C.

TABLE 5

Effects of pH, IgM Concentration and Incubation Time on C_{4a} Generation* in vitro and IgM Antigenic Determinants of IgM Immunoglobulin Concentrates at 50° C.

Incubation	50% IgM pH 4.42		50% IgM pH 4.24		20% IgM pH 4.25	
Time (Min)	C_{4a} μg/ml	IgM mg/ml	C_{4a} μg/ml	IgM mg/ml	C_{4a} μg/ml	IgM mg/ml
0	7.06	37.90	5.45	37.90	5.00	10.07
15	2.76	37.90	1.22	37.90	1.26	10.07
30	2.52	37.90	0.98	35.56	0.88	10.38
45	2.12	37.90	1.03	35.56	0.96	10.38
60	2.08	36.73	0.54	33.27	0.94	9.77
90	1.74	37.90	0.98	33.27		
120	1.20	35.56	0.79	28.81	0.94	8.32
150	1.03	36.73				
180	0.88	37.90	0.56	26.65	0.90	6.1

*Control (no exogenous immunoglobulins) $C4_a$ levels have been subtracted from all reported values.

Pd IgM immunoglobulin concentrates containing 50% IgM at pH 4.42 heated at 50° C. for 3 hours resulted in a decrease in $C4_a$ generation from 7.06 μg/ml to 0.88 μg/ml and fully retained IgM antigenic determinants. Pd IgM immunoglobulin concentrates containing 50% IgM at pH 4.24 heated at 50° C. for 60 minutes caused $C4_a$ generation in vitro to decrease to 0.54 μg/ml and still retained significant IgM antigenic determinant (88%). IgM concentrates containing 20% IgM at pH 4.25 heated at 50° C. for 30 minutes caused $C4_a$ generation to decrease to 0.88 μg/ml with little loss of IgM antigenic determinants and further incubation for 180 minutes did not cause any additional decrease in $C4_a$ generation in vitro but resulted in a loss of IgM antigenic determinants (40%).

In order to further evaluate the effects of heating on immunoglobulin effector functions we assayed antigen binding activity of IgM to Ps. IT4 lipopolysaccharide under various heating conditions. These results are summarized in Table 6.

TABLE 6

Effects of Temperature and Incubation Time on Antigenic Determinants and Specific Antigen Binding Activity of Pd IgM Concentrates.

	Heat		RID IgM	ELISA α Ps IT$_4$ LPS	Specific Activity α LPS
Sample	°C.	Min.	mg/ml	mg/ml	mg/mg IgM
3747-82-E (pH 4.42)	—	—	36.0	0.542	0.015
18107-58-1	62	10	17.42	0.400	0.023
18053-62-6	62	120	6.58	0.040	0.006
18107-62-3	55	30	26.77	0.518	0.019
18107-62-5	55	50	24.61	0.364	0.015
18107-72-9	52	150	35.00	0.483	0.014
18107-72-11	52	210	32.32	0.455	0.014
18107-63-7	50	180	32.38	0.427	0.013
18107-67-15	45	480	35.68	0.604	0.017
18107-70-1	50	180	32.90	0.419	0.013

Pd IgM, IgG concentrates heated at 62° C. for 120 minutes adversely affected the IgM antigenic determinants, resulting in a loss of more than 90% of specific antigen binding activity, and also a 3 fold reduction in specific activity (α LPS/IgM). Samples treated at the lower temperatures all retained significant specific antigen binding activity and non-significant decrease in specific activity.

We next examined what effect hating had on opsonic activity, another important indicator of biologic effector function. These results are summarized in Table 7.

TABLE 7

Effect of Temperature and Incubation Time on Opsonic Activity of 50% IgM concentrate

Incubation	LOG$_{10}$ CFU Reduction of *E. Coli* 050:K1 °C.					
Time (min.)	62	55	50	45	40	37
0	3.12	2.86	2.86	2.86	2.86	2.86
10	0.25	2.81				
20	0.19	1.79	3.23			
40		0.55	3.35			
60	0.17	0.42	3.27			
120		0.51	3.07			
180			2.71			
240			2.19			
300			2.18			
480				3.09	3.21	3.09
5% Guinea Pig Serum Only	0.15	0.41	0.41	0.41	0.41	0.41

Unheated IgM significantly enhanced bacterial killing. IgM, IgG concentrates heated at 62° C. for 10 minutes lost substantial opsonic activity. Concentrates heated at 55° C. had diminished activity at 20 minutes and lost substantial opsonic activity at 40 minutes. Heating at 50° C. slightly reduced opsonic activity over time but substantial opsonic activity still remained at 5 hours.

Heating at temperature between 45°–37° C. did not diminish opsonic activity over hours.

Opsonic activity of the IgM, IgG immunoglobulin concentrate heated at 50° C. for 3 hours was also assessed in a phagocyte chemiluminescence assay against *E. coli* 050:K1, FIG. 5. Heating IgM at 50° C. for 3 hours leaves intact the ability of IgM to promote chemiluminescence and phagocytic killing of the bacteria.

Since IgM, IgG immunoglobulin concentrates heated at 50° C. for 3 hours retained effector functions i.e., opsonophagocytic activity, antigenic binding sites, etc., and demonstrated diminished non-specific complement activation in vitro ($C4_a$ generation), we assessed the cardiovascular effects of this preparation following intravenous infusion in the cynomolgus monkey. This data is summarized in Table 8.

TABLE 8

Acute effect of heat treated IgM, IgG immunoglobulin concentrates on MAP and plasma $C4_a$ anaphylatoxin levels in the monkey (N = 3).

	Time (min.)			
	0	30	60	90
MAP (mmHg)	92 ± 7	85 ± 5	88 ± 9	93 ± 7
$C4_a$ (ng/ml)	85 ± 17	326 ± 102	500 ± 52	685 ± 61

IgM, IgG heated at 50°–51° C. for 3 hours.
Rate 1 mg/kg/min
Dose 50 mg/kg

Severe hypotension was not observed in these monkeys following infusion of the immunoglobulin concentrates and plasma $C4_a$ levels were much diminished compared with animals infused with the unheated IgM preparation (Table 2).

Discussion

The parenteral administration of IgM enriched IgG (IgM, IgG immunoglobulin concentrates) is associated with serious side effects including severe systemic hypotension in the cynomolgus monkey. The mechanism whereby IgM, IgG concentrate infusion elicits these adverse effects is not presently known.

In these experiments, however, we have shown that the ability of various immunoglobulin preparations to induce systemic hypotension is related to their capacity to activate the classical complement pathway. That is, immunoglobulin preparations which activate the classical pathway of complement in vitro, (i.e., pdIgM and heat-treated IgG at neutral pH) elicit systemic hypotension when administered intravenously to the monkey. While immunoglobulin preparations which do not activate the classical pathway of complement in vitro, (e.g., heat-treated pdIgM, native IgG and heat-treated IgG at acid pH) do not elicit any adverse hemodynamic effects when administered intravenously to the monkey.

It therefore appears that the in vitro assessment of complement activation (classical pathway) of various immunoglobulin preparations has predictive value for estimating the capability of these preparations to elicit adverse effects in the monkey. Whether this is a direct cause and effect relationship or these phenomenon are merely temporarily related has not been determined. Furthermore, and of greater importance, we have shown that mild heat-treatment of pdIgM, IgG immunoglobulin concentrates diminishes its potential to non-specifically activate complement in vitro and this terminal process treatment greatly decreases it's ability to induce adverse the cynomolgus monkey.

Since antigenic determinants and specific antigen binding sites are retained with less harsh heat-treatment (at the presently preferred temperature of about 50° C. for 3 hours) it would appear that antibody integrity has not been compromised at these temperatures while non-specific complement activating potential has been dramatically diminished, thus this treatment would result in a much better product.

It has now been demonstrated that IgM-enriched, IgG immunoglobulin concentrates can be heat treated at elevated temperatures for extended periods of time without significant loss of antigenic determinants or specific antigen binding sites. The preparations still retain opsonophagocytic activity while exhibiting dramatically diminished non-specific complement activity. Consequently, through suitable heating temperatures for suitable periods of time at suitable pH, suitable protein concentration and suitable stabilizer, the non-specific complement activity can be diminished in the IgM-enriched, IgG immunoglobulin concentrate product while retaining the antigenic determinants, specific antigen binding sites, specific complement activity when bound to antigen (opsonophagocytic activity) and therapeutic integrity of pdIgM, IgG immunoglobulin concentrates product.

Given the above disclosure, it is thought that variations will occur to those skilled in the art. Accordingly, it is intended that the above disclosure should be construed as illustrative and the scope of the invention should be limited only by the following claims.

We claim:

1. A method of treating an antibody preparation comprising antibodies of the IgM type, the method comprising the step of subjecting the preparation to a gentle heating step at a temperature ranging from 45° C. to 55° C. in an aqueous solution having a pH of 4.0 to 5.0 for at least 10 minutes to minimize any non-specific complement activation without substantial reduction of the specific complement activation activity of the IgM.

2. The method of claim 1 wherein the preparation is heated for about 1 to 3 hours at a temperature of about 50° C.

3. The method of claim 2 wherein the pH is about 4.24 to 4.42.

* * * * *

EXHIBIT 3

IN THE HIGH COURT OF JUSTICE HC 06 C01197

CHANCERY DIVISION

PATENTS COURT

MR JUSTICE PATTEN
FRIDAY THE 27TH DAY OF OCTOBER 2006

SUPREME COURT OF JUDICATURE
31 OCT 2006
ENTERED
CHANCERY CHAMBERS

BETWEEN:

(1) BAXTER HEALTHCARE SA
(2) BAXTER HEALTHCARE PHARMACEUTICAL LIMITED
(3) BAXTER HEALTHCARE LIMITED

Claimants

-and-

(1) BAYER CORPORATION
(2) BAYER HEALTHCARE LLC
(3) TALECRIS BIOTHERAPEUTICS INC

Defendants

CONSENT ORDER

UPON the Solicitors for the Claimants and the Solicitors for the Defendants each agreeing in writing to this Order

AND UPON READING the documents recorded in the court file as having been read

IT IS BY CONSENT ORDERED THAT:

1. It is hereby declared that European Patent (UK) No. 0 764 447 B1 (the "Patent") is and has at all material times been invalid.

2. The Patent be revoked.

3. The Defendants do pay the Claimants their costs of these proceedings, such costs to be assessed if not agreed.

4. The Defendants pay to the Claimants an interim payment of £160,000 on account of the Claimants' costs of these proceedings. The Defendants shall pay said amount within 14 days of the date hereof. The Defendants shall pay interest pursuant to section 35A of the Supreme Court Act 1981 on any sum not paid within 14 days hereof.

Bird & Bird

Bird & Bird

On behalf of the Claimants

Simmons & Simmons

On behalf of the Defendants

[illegible]6 HC 06 C01197

IN THE HIGH COURT OF JUSTICE

CHANCERY DIVISION

PATENTS COURT
MR JUSTICE PATTEN
27TH OCTOBER 2006

BETWEEN:

(1) BAXTER HEALTHCARE SA

(2) BAXTER HEALTHCARE PHARMACEUTICAL LIMITED

(3) BAXTER HEALTHCARE LIMITED

Claimants

-and-

(1) BAYER CORPORATION

(2) BAYER HEALTHCARE LLC

(3) TALECRIS BIOTHERAPEUTICS, INC.

Defendants

CONSENT ORDER

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Solicitors for the Defendants
and copy to Bird & Bird

EXHIBIT 4

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REPLY TO Wilmington Office

July 25, 2006

VIA E-MAIL
Susan M. Spaeth, Esquire
Townsend and Townsend and Crew LLP
379 Lytton Avenue
Palo Alto, CA 94301-1431

RE: Talecris Biotherapeutics, Inc. v. Baxter International, Inc. and Baxter Healthcare Corp., C.A. No. 05-349-GMS

Dear Susan,

Further to our conversation yesterday, Plaintiffs will make the following individuals available for deposition at our offices in Wilmington on the dates indicated below:

September 20, 2006	Bill Zabel
September 26, 2006	William Alonso
September 27, 2006	Clara Schreiner
September 28, 2006	George Baumbach
September 29, 2006	Susan Trukawinski

We continue to await Baxter's confirmation of specific dates for the individuals to be deposed in Austria (week of August 28), Belgium (weeks of September 11 and 18) and Westlake Village (week of August 21). These individuals have been noticed previously and identified as available during the time periods specified for a number of weeks.

Sincerely,

Jeffrey B. Bove

cc: Philip Rovner (via e-mail)
Bradford J. Badke (via e-mail)
Mary W. Bourke

EXHIBIT 5

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and
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August 14, 2006

VIA EMAIL

Jeffrey B. Bove
Connolly Bove Lodge & Hutz LLP
The Nemours Building
1007 North Orange Street
P.O. Box 2207
Wilmington, DE 19891-2207

Re: ***Talecris Biotherapeutics, Inc., et al. v. Baxter International Inc., et al.***
U.S.D.C., Dist. of Delaware, Action No. 05-349-GMS
Our Reference No. 018652-004000

Dear Jeff:

I am writing to you regarding specific deposition issues. In your July 14, 2006 letter to Susan Spaeth, you supplemented your initial disclosures and identified nine witnesses that you intend to produce for depositions. To date, however, we have not received deposition dates for Teresa Blackmon or Tom Rains. Please provide dates when these witnesses will be available for deposition.

Considering the importance of Dr. William Alonso's testimony regarding his alleged invention and his patent, which is at the heart of your lawsuit, we believe it will be necessary to depose him for two consecutive, seven-hour days. Given Susan Trukawinski's involvement with Dr. Alonso's research in support of the patent, we also expect her deposition will require two consecutive, seven-hour days. Please confirm that Baxter can depose both William Alonso and Susan Trukawinski each for two, consecutive seven-hour days.

Thank you for your proposal for Susan Trukawinski's deposition in late September. However, we would like to depose her earlier in September. We propose that her deposition proceed sometime during the week of September 5-8, 2006. Please confirm that Ms. Trukawinski will be available for two consecutive days during that week and please specify which days she will be available.

We also wish to depose Terry Tenbrunsel. Please provide dates when Mr. Tenbrunsel will be available for a deposition.

Additionally, we plan to notice a 30(b)(6) deposition of Talecris/Bayer. We will send out a formal 30(b)(6) Notice of Deposition; however, to expedite the process, following are some of the categories for which we will request documents and deposition testimony:

- Licenses, sales, assignments or offers to license, offers to sell, offers to assign and/or offers to purchase the '191 patent;
- Processes for the manufacture of Gamimune, including information on yield comparisons between Gamimune and other products;
- Processes for the manufacture of Gamunex; including information on yield comparisons between Gamunex and other products and the development of these processes;
- Sales and marketing of Gamunex;
- Sales and marketing of Gamimune;
- Quality assurance/ quality control information for Bayer/ Talecris' immunoglobulin products, including but not limited to, Gamimune and Gamunex;
- Products that Bayer was developing during the time period of the research and experiments for the '191 patent;
- Predecessor immunoglobulin products that Bayer developed prior to Gamimune and Gamunex since 1980;
- Information regarding the Octapharma litigation, including but not limited to positions taken in that case by Octapharma and Bayer, documents exchanged, depositions taken and outcome of the litigation;
- Experiments relating to the '191 patent;
- Information regarding Talecris' supply of plasma.

Baxter may add or modify categories in a subsequent 30(b)(6) Notice of Deposition. In the meantime, however, we request that you begin evaluating the appropriate witness(es) and their availability for deposition and provide us with available dates. I look forward to hearing from you.

Very truly yours,

Priya Sreenivasan

Priya Sreenivasan

PS/AMR:lt

cc: Philip A. Rovner, Potter Anderson & Corroon LLP (via e-mail)
Jim Badke, Ropes & Gray LLP (via e-mail)

60837442 v1

EXHIBIT 6

THIS EXHIBIT HAS BEEN

REDACTED IN ITS ENTIRETY

EXHIBIT 7

THIS EXHIBIT HAS BEEN REDACTED IN ITS ENTIRETY

EXHIBIT 8

THIS EXHIBIT HAS BEEN

REDACTED IN ITS ENTIRETY

EXHIBIT 9

THIS EXHIBIT HAS BEEN

REDACTED IN ITS ENTIRETY

**IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE**

CERTIFICATE OF SERVICE

I, Philip A. Rovner, hereby certify that on December 4, 2006, the within document was filed with the Clerk of the Court using CM/ECF which will send notification of such filing(s) to the following; that the document was served on the following counsel as indicated; and that the document is available for viewing and downloading from CM/ECF.

BY HAND DELIVERY AND E-MAIL

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I hereby certify that on December 4, 2006 I have sent by E-mail and Federal Express the foregoing documents to the following non-registered participants:

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